Carbohydrate Polymers **15** (1991) 79–87

Preparation and Molecular Characterization of Carboxymethylglucan Fractions

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(Received 1 February 1990; accepted 1 March 1990)

ABSTRACT

*Sodium salt of carboxymethylglucan (CMG-Na), prepared from beta-*D*-glucan isolated from baker's yeast* Saccharomyces cerevisiae, *was fractionated from its aqueous solution by stepwise precipitation using acetone. The fractions obtained were characterized by gel permeation chromatography (GPC), light scattering, and viscometry. The results of molecular characterization of the CMG-Na fractions are discussed in the possible 'structural inhomogeneity' of the investigated sample.*

INTRODUCTION

High-molecular-weight glucans belong to the most widely spread biopolymers. When introduced into the living body, they have various biological effects, such as immunostimulating effect, antitumor activity (Di Luzio *et al.*, 1979), etc. Schizophyllan, lentinan, curdlan, pachymaran, and others are beta-1,3-D-glucans with single beta-1,6-linked glucopyranose residues, the number of which varies. The molecu-

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Carbohydrate Polymers 0144-8617/91/$03.50

lar weight of water-soluble beta-D-glucans, higher than 1×10^5 (Kojima *et al.*, 1986), along with the triple-helical structure of their polymer chain (Norisuye *et al.*, 1980, Yanaki *et al.*, 1980, Kashiwagi *et al.*, 1981, Sato *et al.*, 1983), are considered to be the key properties responsible for the biological activities (Hamuro *et al.*, 1971, Chihara, 1984*a*, *b*; Maeda *et al.*, 1988).

The beta-D-glucan isolated from the cell walls of the baker's yeast *Saccharomyces cerevisiae* is a branched polysaccharide with beta-1,3- and a small amount of beta-1,6-glucosidic linkages (Kogan *et al.*, 1988) insoluble in water. So, it is important for an application to prepare its water-soluble derivative. The heterogeneous etherification of the particulate beta-D-glucan with monochloroacetic acid in alkaline medium yields a water-soluble derivative, i.e. the sodium salt of carboxymethyl-(1→6)-beta-D-gluco-(1→3)-beta-D-glucan (CMG-Na). The CMG-Na potentiates an antibacterial effect of antibiotics (J. Navarová *et al.*, unpublished data) and shows immunostimulating and radioprotective effects (A. Líšková *et al.*, unpublished data).

This paper deals with fractionation and molecular characterization of the fractions of carboxymethylglucan sodium salt by means of gel permeation chromatography (GPC), light scattering (LS) and viscometry.

MATERIALS AND METHODS

Materials

The following chemicals were used: acetone, diethyl ether, $NaH_2PO_4 \times 2H_2O$, $Na_2HPO_4 \times 12H_2O$, NaCl, phenol, 96% H_2SO_4 (Lachema Brno, Czechoslovakia, all of chemical grade). Sepharose 2B-CL and dextrans of series T ($\bar{M}_w = 3{\cdot}95 \times 10^4$, $1{\cdot}67 \times 10^5$, $4{\cdot}96 \times 10^5$) were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Hydroxyethylstarches ($\bar{M}_w = 5{\cdot}38 \times 10^4$, $1{\cdot}28 \times 10^5$, $1{\cdot}95 \times 10^5$, $3{\cdot}98 \times 10^5$, $9{\cdot}37 \times 10^5$, $1{\cdot}92 \times 10^6$) were kindly supplied by Dr Kirsti Granath (Pharmacia Fine Chemicals, Uppsala). The crude sample of CMG-Na was prepared as follows. Glucan was isolated from the cell walls of yeast by Masler and Šandula (Kogan *et al.*, 1988). Subsequently, 100 g glucan was alkalinized in 124 ml aqueous NaOH (30 g/100 ml) and 1250 ml isopropyl alcohol for 1 h at 10°C. After alkalinization, glucan reacted with sodium salt of monochloroacetic acid (143 g in 140 ml of water) for 2 h at 70°C. Then an excess of NaOH was neutralized and salts were removed by dialysis. CMG-Na was dried, dissolved in water, filtered and lyophilized. The yield of CMG-Na with the degree of substitution of 0·91 was 95% and the residual water level was 15%.

Fractionation

The crude sample of CMG-Na was fractionated from aqueous NaCl (0·5 g/100 ml) solution (polymer concentration 0·4 g/100 ml) using acetone as a precipitant. The main fractions obtained (I, II, III) were reprecipitated, thoroughly washed with acetone and diethyl ether, and dried at laboratory temperature. The middle fraction (II) was refractionated and a series of subfractions were isolated (Fig. 1). The main fractions as well as the subfractions had residual water levels of about 16%.

Gel permeation chromatography

Gel permeation chromatography (GPC) was performed in a glass column (length 215 mm, i.d. 25 mm) packed with Sepharose 2B-CL, particle size 60–200 μm, using phosphate buffer 50 mmol/liter, pH 7·53

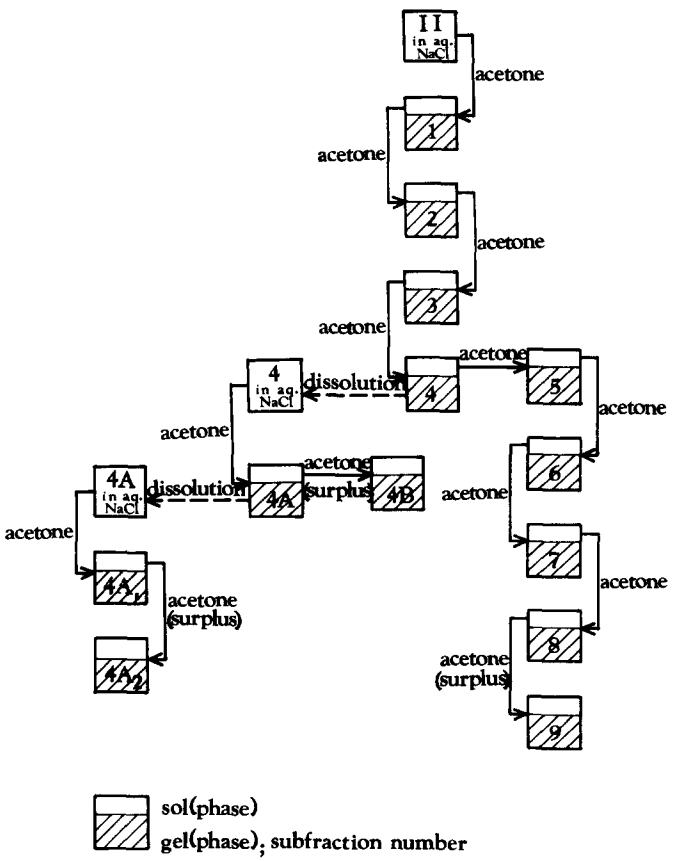

Fig. 1. Subfractionation of the CMG-Na fraction II.

as the mobile phase. The elution rate was 0·5 ml/min, the concentration of the applied sample was 2 mg/ml in the mobile phase and the injected sample volume was 1 ml. The concentration of the polymer in the effluent (3-ml portions) was determined by the phenolsulfuric acid method (Dubois *et al.*, 1956). The separation efficiency of the used GPC column was determined with a set of dextrans and hydroxyethylstarches. The characteristic sample elution volume, V_e, was that corresponding to the maximum of its chromatographic curve.

Light scattering

Weight-average molecular weight, $\bar{M}_w$, was measured by light scattering (LS) using a Sofica instrument equipped with an He–Ne laser (vertically polarized, $\lambda = 633$ nm) in an angular range of 30–150°. Polymer solutions of several concentrations (minimum three) in aqueous NaCl (0·1 mol/liter) were optically cleaned by centrifugation at 10 000 rev/min on a preparative Spinco ultracentrifuge directly in LS cells using a swinging rotor SW 25·1. The data were treated by the conventional Zimm method. The refractive index increment (0·130 ml/g) was measured with a Brice-Phoenix differential refractometer.

Viscometry

Viscosity measurements were performed with a Seide and Deckert viscometer. Intrinsic viscosity values, $[\eta]$, in milliliters per gram, were determined from at least four concentrations, and evaluated using the equations:

$$\frac{\eta_{sp}}{c} = [\eta] + k_H[\eta]^2 c \tag{1}$$

and

$$\frac{\ln \eta_{rel}}{c} = [\eta] - k'_H[\eta]^2 c \tag{2}$$

where η_{sp} and η_{rel} are specific and relative viscosities, respectively, and k_H and k'_H are Huggins' constants ($k_H + k'_H = 0{\cdot}5$).

RESULTS AND DISCUSSION

Fractionation of the CMG-Na sample was carried out by using a similar procedure as for the hydroxyethylcellulose fractionation (Mislovičová *et*

al., 1985). Several organic precipitants were tested for CMG-Na fractionation; acetone proved to be the most suitable one. First, three fractions (I, II and III) were separated (Table 1) with the total yield of 84% (87·2% parallel fractionation). The chromatograms of the fractions (Fig. 2) from several fractionations were virtually identical.

The subfractionation scheme of fraction II is presented in Fig. 1, the yields are given in Table 1. The overall subfractionation yield was 87·4%. All fractions were characterized by GPC, with the exception of subfractions 1 and 5 due to their small amounts. The V_e positions of the main peak of the subfractions were in the range 58·5–82·5 ml (Table 1). The samples showing a single-peak chromatogram seemed to be suitable for the $\bar{M}_w$ determination by LS (Table 1).

To determine the molecular weight distribution and molecular weight averages, the GPC column had to be calibrated not only in terms of the $\bar{M}_w$–V_e dependence (Fig. 3), but also in terms of the intensity of longitudinal diffusion (Tung, 1966). To evaluate the spreading factor h, fractions of dextran and hydroxyethylstarch were used. The method for the determination of the h values and that for evaluation of the h–V_e dependence, have been described by Mislovičová *et al.* (1985) and Šoltés *et al.* (1980).

The calibration dependence $\bar{M}_w$ versus V_e for CMG-Na, and the dependence h versus V_e, along with the iteration program of Chang and Huang (Chang & Huang, 1969), were applied to calculate the corrected chromatograms and the molecular-weight distribution, as well as the number- and weight-average molecular weights of the individual fractions and subfractions. The calculated $\bar{M}_w$ and $\bar{M}_w/\bar{M}_n$ values for the subfractions and the fractions II and III are given in Table 1. With the exception of subfraction 9, $\bar{M}_w$ values determined by GPC and by LS are in a good agreement. The $\bar{M}_w/\bar{M}_n$ of the studied polymer samples range from 1·2 to 1·8.

The $[\eta]$ versus $\bar{M}_w$ dependences for selected samples (Fig. 4) can be expressed as:

$$[\eta] = 0{\cdot}270 \,.\, \bar{M}^{0{\cdot}46}_{w(GPC)} \qquad (r = 0{\cdot}9337) \tag{3}$$

valid for samples 6, $4A_2$, 7, 4B, and 8, and

$$[\eta] = 2{\cdot}580 \,.\, \bar{M}^{0{\cdot}28}_{w(LS)} \qquad (r = 0{\cdot}9685) \tag{4}$$

valid for samples $4A_2$, 4B, and 8 (r being the correlation coefficient). The subfractions 2, 3 and the fraction III lie rather far from the given dependences (3) and (4) (Fig. 4). Very low values of $[\eta]$ and relatively high values of Huggins' constant (0·512–1·604) point to different hydrodynamic behavior of these fractions with respect to that of the

TABLE 1

Molecular Characteristics of the Carboxymethylglucan Fractions. For the Symbols see the Text. Yield Values in the Brackets are from the Parallel Fractionation

Fraction	*% of Yield*	*V_e (ml) of*		$\bar{M}_{w(LS)}$	$\bar{M}_{w(GPC)}$	$\bar{M}_w/\bar{M}_{n(GPC)}$	$[\eta]\times10^{-2}$ *(ml/g)*	k_H	
		Main peak	*Side peak*					*a*	*b*
I	9·3 (11·1)	63·0	97·5, 116·5						
II	73·1 (71·4)	67·5			$8{\cdot}84\times10^5$	1·55	0·966	0·278	0·315
III	1·6 (4·7)	91·5	114·0	$3{\cdot}38\times10^5$	$3{\cdot}81\times10^5$	1·23	0·140	1·604	1·225
Total	84·0 (87·2)								
1	1·1								
2	3·0	64·5	52·5, 94·5		$1{\cdot}03\times10^6$	1·83	0·245	0·584	0·512
3	5·2	61·5	91·5		$8{\cdot}65\times10^5$	1·76	0·197	1·075	0·813
$4A_1$	11·1	61·5	94·5		$9{\cdot}31\times10^5$	1·47	0·980	0·160	0·259
$4A_2$	20·6	58·5		$9{\cdot}64\times10^5$	$9{\cdot}11\times10^5$	1·56	1·193	0·341	0·348
4B	9·7	67·5		$7{\cdot}56\times10^5$	$7{\cdot}37\times10^5$	1·65	1·074	0·281	0·323
5	1·2								
6	8·5	64·5	31·5, 91·5		$9{\cdot}59\times10^5$	1·61	1·176	0·251	0·321
7	7·7	64·5	70·5		$8{\cdot}17\times10^5$	1·46	1·163	0·246	0·309
8	10·5	73·5		$5{\cdot}40\times10^5$	$6{\cdot}82\times10^5$	1·36	1·012	0·184	0·285
9	8·8	82·5		$4{\cdot}20\times10^6$	$5{\cdot}77\times10^5$	1·43	0·657	0·320	0·338
Total	87·4								

k_H: *a* — calculated by using the eqn (1), *b* — calculated by using the eqn (2).

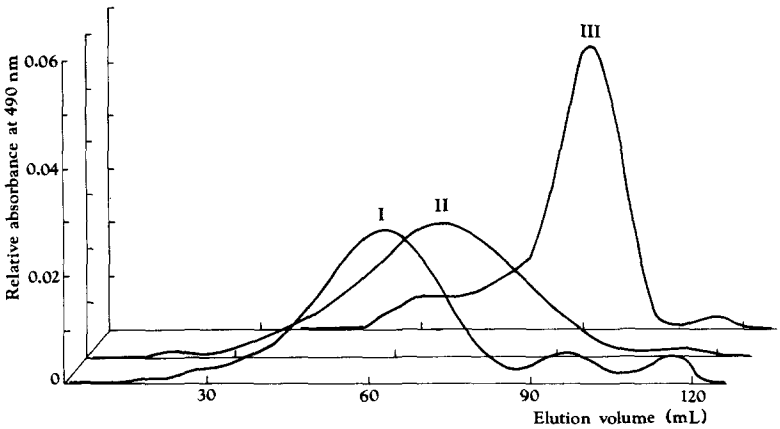

Fig. 2. Normalized GPC-curves of CMG-Na fractions I, II, and III.

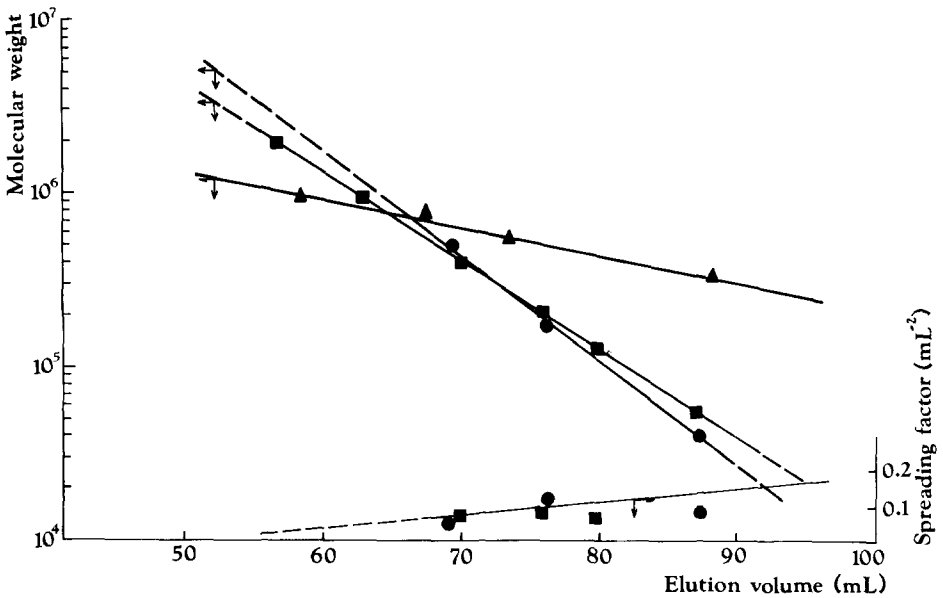

Fig. 3. Calibration of the GPC column. ●, Dextran; ■, hydroxyethylstarch; ▲, CMG-Na fraction (CMG-Na sample No. 9 not included).

other fractions. The k_H values of those other fractions (0·160–0·348) are typical for linear and low-branched polymers ($0{\cdot}2 \leq k_H \leq 0{\cdot}6$). The fact that the subfractions 9, $4A_1$, as well as 2, 3, do not fit the dependences (3) and (4) suggests that they differ from other fractions not only in their molecular weights but also in their 'structural inhomogeneity' — i.e., in the ratio of 1,3- and 1,6-linkages, in the branching frequency of the main

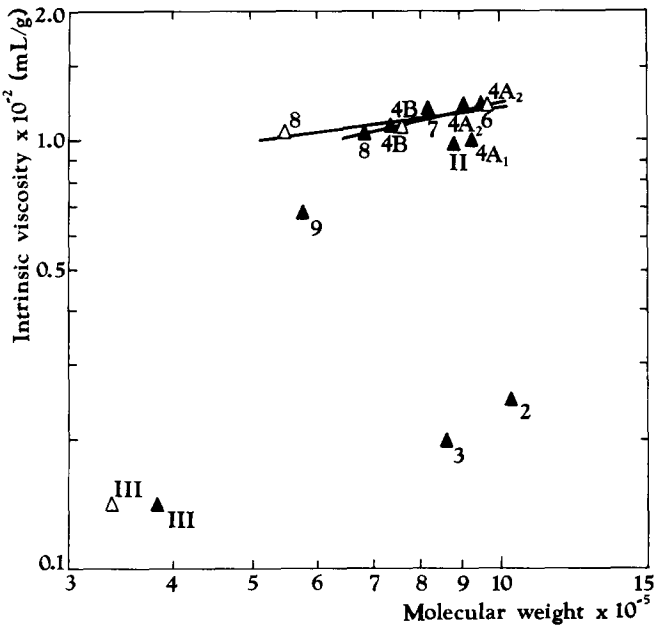

Fig. 4. Intrinsic viscosity versus molecular weight of CMG-Na fractions. ▲ $\bar{M}_w$ from GPC, △ $\bar{M}_w$ from LS.

polymer chain, and in the degree of substitution with carboxymethyl groups (in the consequence of a heterogeneous derivation).

CONCLUSIONS

The fractionation of the CMG-Na sample is fairly reproducible in terms of the yield, $[\eta]$ values, and the shape of the GPC elution curve of the fractions which differ not only in their $\bar{M}_w$ but also in the 'structural inhomogeneity'. However, since the fractions and subfractions under study may differ in their structure — which controls their hydrodynamic behavior — the molecular parameters determined by the GPC method may have only an apparent character.

ACKNOWLEDGEMENTS

We thank Drs Masler, Šandula, and Pastýr (from Institute of Chemistry, Slovak Academy of Sciences, Bratislava) for preparation of the CMG-Na sample.

REFERENCES

Chang, K. S. & Huang, R. Y. M. (1969). *J. Appl. Polym. Sci.*, **13**, 1459–71.
Chihara, G. (1984*a*). Estratto dalla rivista EOS. *Edizione sigma-tau*, **IV** (2), 85–96.
Chihara, G. (1984*b*). *Tissue Culture and RES*, ed. P. Röhlich & E. Bácsy. Publishing House of the Hungarian Acad. of Sci., Budapest, pp. 179–91.
Di Luzio, N. R., Williams, D. L., McNamee, R. B., Edwards, B. F. & Kitahama, A. (1979). *Int. J. Cancer*, **24**, 773–9.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). *Anal. Chem.*, **28**, 350–6.
Hamuro, J., Maeda, Y. Y., Arai, Y., Fukuoka, F. & Chihara, G. (1971). *Chem. Biol. Interactions*, **3**, 69–71.
Kashiwagi, Y., Norisuye, T. & Fujita, H. (1981). *Macromolecules*, **14**, 1220–25.
Kogan, G., Alföldi, J. & Masler, L. (1988). *Biopolymers*, **27**, 1055–63.
Kojima, T., Tabata, K., Itoh, W. & Yanaki, T. (1986). *Agric. Biol. Chem.*, **50**, 231–2.
Maeda, Y. Y., Watanabe, S. T., Chihara, Ch. & Rokutanda, M. (1988). *Cancer Res.*, **48**, 671–5.
Mislovičová, D., Gemeiner, P. & Šoltés, L. (1985). *Collect. Czech. Chem. Commun.*, **50**, 1335–40.
Norisuye, T., Yanaki, T. & Fujita, H. (1980). *J. Polym. Sci.*, **18**, 547–58.
Sato, T., Norisuye, T. & Fujita, H. (1983). *Macromolecules*, **16**, 185–9.
Šoltés, L., Berek, D. & Mikulášová, D. (1980). *Colloid Polym. Sci.*, **258**, 702–10.
Tung, L. H. (1966). *J. Appl. Polym. Sci.*, **10**, 375–85.
Yanaki, T., Norisuye, T. & Fujita, H. (1980). *Macromolecules*, **13**, 1462–6.